Rapid detection of ESBL, carbapenemases, MRSA and other important resistance phenotypes within 6-8 h by automated disc diffusion antibiotic susceptibility testing

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Rapid detection of ESBL, carbapenemases, MRSA and other important resistance phenotypes within 6–8 h by automated disc diffusion antibiotic susceptibility testing

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Background: In principle, automated systems allow rapid reading of disc diffusion AST (rAST) within 6–8 h.

Objectives: This study analysed whether rAST can discriminate resistance phenotypes such as ESBL, carbapenemases and MRSA/methicillin-resistant *Staphylococcus epidermidis* from WT populations. We describe species–drug combinations that may require clinical breakpoint adaptions for early reading due to zone diameter changes during the incubation period.

Methods: In total, 1852 clinical strains [*Escherichia coli* ($n = 475$), *Klebsiella pneumoniae* ($n = 375$), *Enterobacter cloacae* ($n = 301$), *Staphylococcus aureus* ($n = 407$) and *S. epidermidis* ($n = 294$)] were included in this study comprising WT populations and important resistance phenotypes, e.g. ESBL, carbapenemases and MRSA. We assessed (i) separation of resistance phenotypes and WT populations after 6, 8 and 12 h as compared with the 18 h standard, and (ii) diameter changes of WT populations and associated putative epidemiological cut-offs during the incubation period. Disc diffusion plates were automatically streaked, incubated and imaged using the WASPLab^{TM} system.

Results and conclusions: We demonstrated that important resistance phenotypes could reliably be separated from WT populations at early reading times for the most prevalent bacterial pathogens encountered in the clinical laboratory. Current AST expert rules and algorithms for identification of resistance mechanisms can readily be applied for rAST, e.g. EUCAST recommended rules for detection of ESBL, AmpC, carbapenemases and MRSA/methicillin-resistant *S. epidermidis*. However, several species–drug combinations may require clinical breakpoint adaptations when using rAST as the diameter, and hence the epidemiological cut-off, changes during the incubation period.

Introduction

Detection of important antibiotic resistance mechanisms such as ESBL, AmpC β-lactamases, carbapenemases, MRSA or inducible erm_{MLS} (iMLS) is critical to ensure a successful clinical outcome.[1–5] Rapid implementation of effective, targeted antibiotic treatment significantly improves clinical outcome and reduces mortality.[6–8] Automated microdilution AST provides results within 6–12 h but carries several disadvantages such as non-flexible drug panels, few drug concentrations tested, minimal detection of synergism/antagonism phenomena and a comparably low sensitivity/specificity for the detection of important resistance mechanisms such as ESBL, carbapenemases or iMLS.[9–11] Molecular detection of resistance determinants is rapid and focused on specific genetic targets, but accurate coverage of the most relevant resistance genes is a laborious task. In addition, the presence of a gene may not correlate with phenotypic expression and off-panel or emerging resistance determinants are readily missed by molecular assays.[12]

Disc diffusion is an accurate, reliable and highly standardized AST method with the advantages of low consumable costs and flexible drug panels. Procedures to detect reliably the important resistance mechanisms and their associated phenotypes using disc diffusion AST have been established. Current disc diffusion based on EUCAST and CLSI guidelines requires incubation times of 16–18 h for most species–drug combinations.[5,13–15]

We recently demonstrated that early reading of disc diffusion is in principle possible by using automated systems.[16] This study

aimed to (i) analyse the potential of rapid disc diffusion AST (rAST), i.e. early zone diameter reading at 6–12 h, to discriminate important resistance phenotypes from WT populations, and (ii) to describe species–drug combinations for which clinical breakpoint (CBP) changes are necessary for early reading due to changes of zone diameters during the incubation period. Diameter changes of the WT result in shifts of corresponding epidemiological cut-offs (ECOFFs), which are a critical parameter in CBP setting.[17]

Methods

Clinical isolates

Study isolates were selected to cover a broad range of inhibition zone diameters (6–40 mm) for each species/drug combination tested (see Figure S1, available as Supplementary data at *JAC* Online). In particular, critical isolates close to the CBPs were included. All non-duplicate clinical strains included in this study were isolated over a 3 year period from 2013 to 2016 in the clinical microbiology laboratory of the Institute of Medical Microbiology, University of Zurich. Isolates of the same species were considered duplicate(s) if they (i) originated from the same patient, and (ii) showed one major and two minor differences in AST interpretation at maximum. The following numbers of clinical isolates were tested: *Escherichia coli* ($N = 475$), *Klebsiella pneumoniae* ($N = 375$), *Enterobacter cloacae* ($N = 301$), *Staphylococcus aureus* ($N = 407$) and *Staphylococcus epidermidis* ($N = 294$).

Quality control strains

To control for methodological precision, *E. coli* ATCC 25922 and *S. aureus* ATCC 29213 EUCAST quality control strains were tested daily from individual fresh sub-cultures and individually prepared McFarland 0.5 standards. Interpretation was done according to EUCAST QC tables, version 6.1.[18] Quality control ranges and targets were met during this study (data not shown).

Definition of phenotypes

Resistance phenotypes and the WT populations were defined as per criteria given in Table S1. Assignment of phenotypes was done prior to this study based on independent disc diameter measurements generated by using the EUCAST recommended method on Mueller–Hinton II agar (Becton-Dickinson, Franklin Lakes, NJ, USA), with antibiotic discs from i2a (i2a, Montpellier, France), and applying EUCAST CBPs.[19] Inhibition zone diameters were recorded using the Sirscan/Sirweb system (i2a). Phenotypic screening and confirmation for the detection of AmpC, ESBL and carbapenemases by combination disc testing was performed as previously described.[13–15]

Automated susceptibility testing

Susceptibility testing was performed as described previously according to EUCAST guidelines version 6.0,[19] which are essentially the same as that of CLSI 2016.[16,20] In brief, bacterial suspensions were manually adjusted to 0.5 McFarland and processed within 15 min. Mueller–Hinton II agar plates (Oxoid Limited, Basingstoke, UK) were processed in the fully automated WASP™ (Copan Italia, S.p.A., Brescia, Italy), i.e. plates were each inoculated with 60 μL of the bacterial suspension and automatically streaked. Antibiotic discs of a single production lot (Oxoid Limited) were placed using a standard distributor, which was handled by the WASP™ robot immediately after plate streaking. Subsequently, plates were automatically transported to and incubated in a WASPLab™ incubator (Copan) at 36±2°C in ambient air. Images were taken after 6, 8, 12 and 18 h of incubation under continuous temperature conditions. Diameter measurements were automatically done by the WASPLab™ reading software (Copan) and were, if necessary, adjusted on-screen by an experienced technician.

Table 1. Early detection of major resistance mechanisms by screening drugs

Resistance mechanisms/ organism groups	Screening drug	Applicable[a] at 6/8 h	Applicable[a] at 18 h
Enterobacteriaceae			
ESBL	ceftriaxone	yes	yes
	cefpodoxime	yes	yes
AmpC	cefoxitin	yes	yes
carbapenemases (general)	meropenem	yes	yes
OXA-48-like enzymes	temocillin	yes	yes
quinolone resistance (high level)	norfloxacin	yes	yes
Staphylococci			
PBP2a	cefoxitin	yes	yes
quinolone resistance (high level)	norfloxacin	yes	yes

[a]Applicability refers to a sensitivity and specificity of at least 0.95.

Statistical analyses

All statistical analyses were performed using R, version 3.2.3.[21] The R package pROC, version 1.8, was used to calculate areas under the receiver operating characteristic curve.[22]

Results

Detection of major resistance mechanisms

In the following, the terms 'well-separated/well-discriminative' indicate separation of WT and non-WT populations with a sensitivity and specificity of at least 0.95 (shown by non-overlapping boxes in Figure S1). 'Fully separated/discriminative' means separation with a sensitivity and specificity of 1 (whiskers in Figure S1 do not overlap). 'Not separable' indicates separation of WT and non-WT population with sensitivity and specificity both >0.95 is not possible (overlapping boxes in Figure S1).

Detection of major resistance mechanisms was readily possible after 6–8 h using the same screening drugs that are used for conventional 18 h incubation AST. ESBL, AmpC, carbapenemases, MRSA and quinolone resistance were reliably detected at early timepoints using EUCAST- and CLSI-recommended marker antibiotics, i.e. with sensitivity and specificity of at least 0.95 (see Table 1).

Change of WT zone diameters and discrimination of WT from non-WT during the incubation period

WT zone diameter changes during the incubation period were dependent on species–drug combinations: for 34 of 44 enterobacterial species–drug combinations (77.3%), the fifth percentile of the WT zone diameters (used as a surrogate for the ECOFF, see Figure 1) remained stable during the incubation period, i.e. it showed an absolute diameter change of ≤2 mm between 6 h and 18 h. For 7

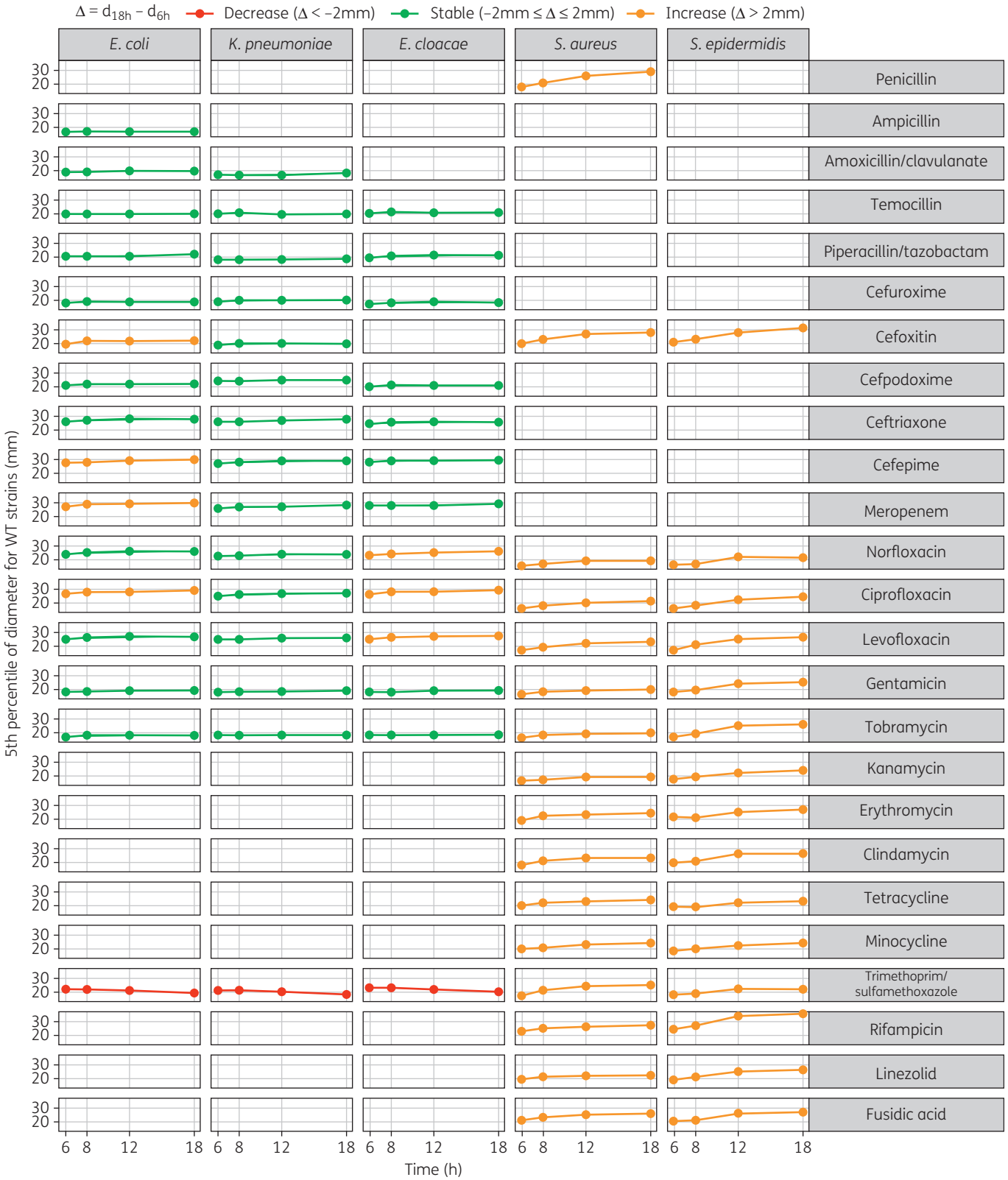

Figure 1. Change of WT zone diameters and putative ECOFFs during the incubation period. Graphs depict the fifth percentile of the WT population. This value is used as surrogate for the ECOFF. Increasing values (yellow lines) or decreasing values (red lines) depict an absolute diameter change of >2 mm between 6 h and 18 h. Stable values (absolute diameter change of ≤2 mm between 6 h and 18 h) are displayed in green.

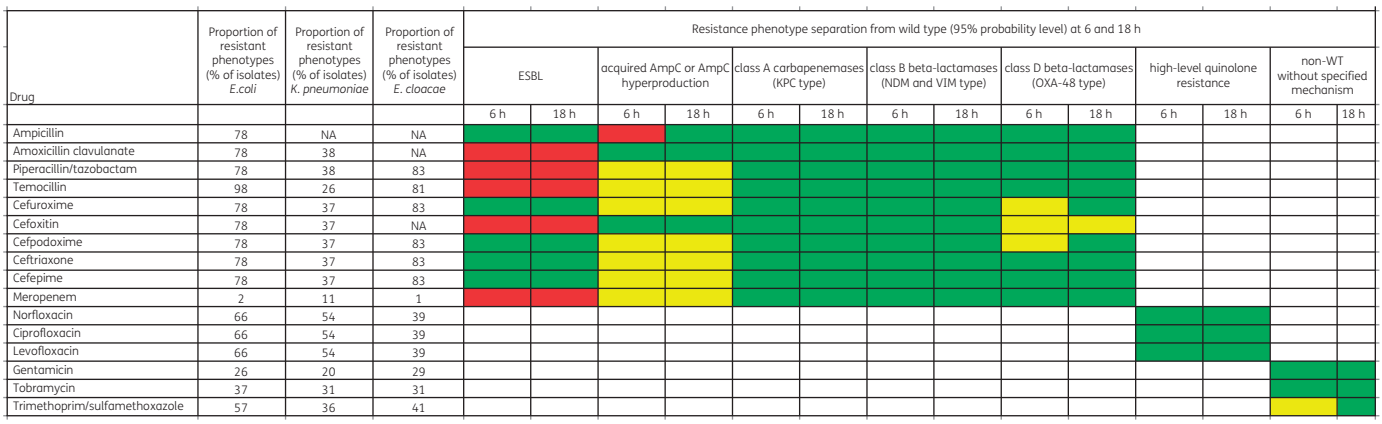

Figure 2. Separation of non-WT and WT populations at 6 h versus 18 h of incubation: Enterobacteriaceae. Green boxes, WT and non-WT populations were well separated (i.e. separation with sensitivity and specificity at least 0.95 is possible); red boxes, WT and non-WT populations were not separated (i.e. separation with sensitivity and specificity at least 0.95 is not possible); yellow boxes, separability of WT and non-WT populations differs among species; proportion of resistant phenotypes: according to the EUCAST recommended method on Mueller–Hinton II agar; NA, not applicable.

of 44 enterobacterial species–drug combinations, i.e. 15.9%, the ECOFF increased by >2 mm. For trimethoprim/sulfamethoxazole we observed a decrease of the ECOFF during the incubation period (3 of 44 combinations, i.e. 6.8%). In contrast, we observed increasing ECOFF for staphylococci irrespective of the drug analysed (Figure 1).

β-Lactams

Enterobacteriaceae

Susceptibility to ampicillin in principle discriminated β-lactamase producers from the WT independently from the reading time with readability ranging from 96% to 100% (accounts for *E. coli* only as *K. pneumoniae* and *E. cloacae* harbour chromosomal SHV and AmpC enzymes, respectively, Figure 2 and Figure S1). ESBL producers were well separated from the WT at 6–8 h by cefpodoxime or ceftriaxone diameters independent of the Enterobacteriaceae species. Cefoxitin was discriminative for the presence of acquired AmpC β-lactamases in *E. coli* and *K. pneumoniae*. For amoxicillin/clavulanate, piperacillin/tazobactam, cefuroxime and cefepime, no differences in separability were detected between early and 18 h reading times for the β-lactamase phenotypes. Meropenem susceptibility at early reading times separated class A, B and D carbapenemases effectively, i.e. KPC, NDM, VIM or OXA-48 producers in the three Enterobacteriaceae species analysed. Temocillin was found to detect OXA-48 enzymes at 6–8 h since WT and OXA-48-positive populations appeared fully separated. In general, β-lactam zone diameters showed a mixed pattern of stable and increasing zones (Figure 1).

Staphylococci

Benzylpenicillin separated the majority of *blaZ* penicillinase-producing *S. aureus* from the WT well at 8 h, while populations were not separated after 6 h (Figure S1). MRSA were well separated at 6–8 h from methicillin-susceptible *S. aureus* by cefoxitin susceptibility, *S. epidermidis* PBP2a-positive non-WT and *S. epidermidis* WT populations were not separable at any reading times.

Fluoroquinolones

Enterobacteriaceae

Fluoroquinolone-resistant non-WT populations were well-separated from the corresponding WT populations at 6 h and fully separated at 8 h for all fluoroquinolones tested and all Enterobacteriaceae species (Figure 2 and Figure S1). Zone diameters increased in all Enterobacteriaceae species, except for *K. pneumoniae* with levofloxacin, for which stable inhibition zones were observed (Figure 1).

Staphylococci

Norfloxacin was fully discriminative for fluoroquinolone-resistant non-WT populations from WT *S. aureus* at 6–8 h and for *S. epidermidis* at 8 h (Figure 3 and Figure S1).

Aminoglycosides

Enterobacteriaceae

Non-WT and WT populations were separated well at 6–8 h in all Enterobacteriaceae species tested (Figure 2 and Figure S1), and WT zone diameters remained stable during the incubation period for all species (Figure 1).

Staphylococci

Non-WT and WT populations were well discriminated at 6–8 h in *S. aureus* and *S. epidermidis*.

Tetracyclines

Staphylococci

The tetracycline non-WT was well-discriminated from the WT at 6–8 h in *S. aureus* but was not separable in *S. epidermidis*. The minocycline non-WT and WT populations were not separable at 6–8 h for *S. epidermidis* and *S. aureus* (Figure 3 and Figure S1). Tigecycline non-WT staphylococcal isolates were not available in this study.

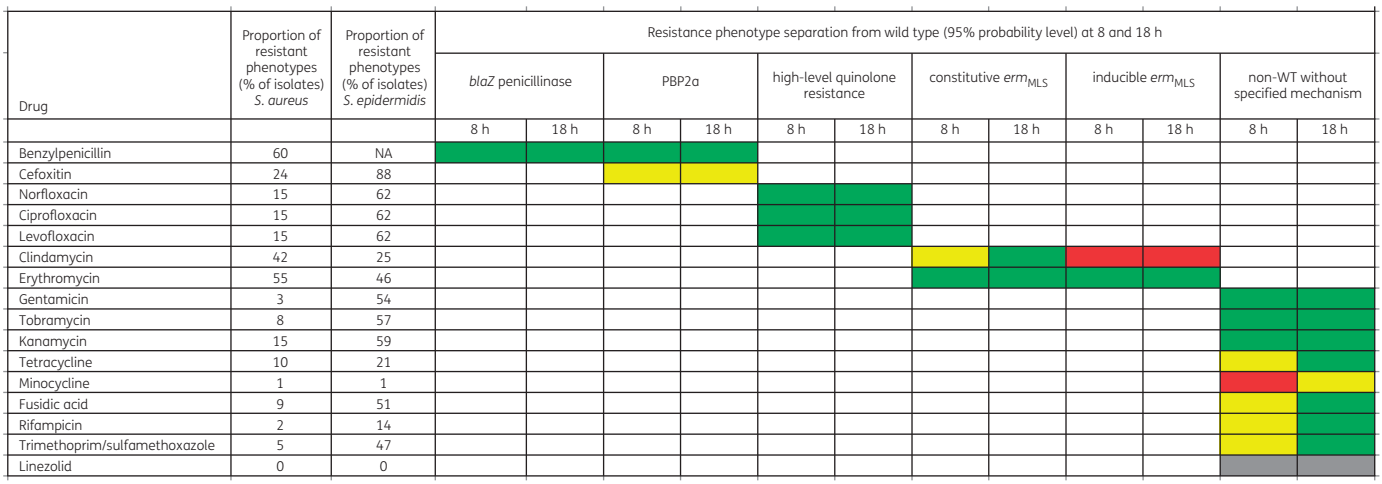

Figure 3. Separation of non-WT and WT populations at 8 h versus 18 h of incubation: staphylococci. For staphylococci, the 8 h reading was selected for comparison with 18 h readings as readability was higher at 8 h (see Figure S1). Green boxes, WT and non-WT populations were well separated (i.e. separation with sensitivity and specificity at least 0.95 is possible); red boxes, WT and non-WT populations were not separated (i.e. separation with sensitivity and specificity at least 0.95 is not possible); yellow boxes, separability of WT and non-WT populations differs among species; grey boxes, non-WT populations not available; proportion of resistant phenotypes according to the EUCAST recommended method on Mueller–Hinton II agar; NA, not applicable.

MLS drugs

Staphylococci

Erythromycin was well to fully discriminative for the constitutive macrolide lincosamide streptogramin (MLS) and iMLS phenotype for all reading times and both staphylococcal species analysed (Figure 3 and Figure S1). For clindamycin, the constitutive MLS non-WT of *S. aureus* was well-separated from the WT at all reading times. In *S. epidermidis* well-separated populations were detected after 18 h only. The iMLS phenotype could not be discriminated by diameter values at all reading times but induction phenomena (D-shape test) were readily detected at early reading times.

Miscellaneous drugs

Enterobacteriaceae

Trimethoprim/sulfamethoxazole non-WT populations were well-separated from the WT at all reading times for the Enterobacteriaceae (except for *K. pneumoniae* at 6 h, see Figure 2 and Figure S1).

Staphylococci

Trimethoprim/sulfamethoxazole and rifampicin non-WT populations were well-separated from the WT at all reading times for *S. epidermidis*. For *S. aureus* discrimination was possible after 18 h only. Fusidic acid non-WT populations were discriminated from the WT at early (8 h) and late reading times for *S. epidermidis* and *S. aureus* (Figure 3 and Figure S1).

Discussion

rAST allowed the timely detection of important resistance phenotypes in the bacterial species that are most frequently encountered in the clinical laboratory. Rapid detection of major resistance mechanisms was readily possible for important drug classes used in patients with life-threatening infections (Figures 1 and 2, Table 1). Basically the same expert rules used for the detection of important resistance mechanisms at 18 h can be applied to rAST, e.g.: (i) cefpodoxime and ceftriaxone were good predictors for the presence of ESBL; (ii) cefoxitin was a good predictor for the presence of acquired AmpC type β-lactamases; (iii) meropenem was a sensitive marker for the presence of any type of carbapenemase; (iv) norfloxacin was a good predictor of fluoroquinolone resistance; (v) cefoxitin was a good marker for MRSA detection; and (vi) iMLS-based clindamycin resistance required additional parameters such as the D-shape test to be detected.[5,13–15,18,20,23] Based on our analyses, the non-WT population zone diameter values are well separated at 6–8 h from WT population diameters for the most important drug classes and resistance phenotypes. In principle, this will allow for reliable resistance phenotype prediction at early timepoints.

Of note, the WASPLab™ automated incubators in the setting of our laboratory did not allow separation of the primary culture plates from the disc diffusion plates. This limitation could be overcome by dedicating a specific incubator to 35°C for disc diffusion AST. However, this seems impractical for most laboratories due to limited space and for cost reasons. We therefore applied an incubation temperature of 36°C as a compromise, but ran EUCAST QCs daily to ensure calibration to EUCAST QC ranges. EUCAST mainly recommends incubation at 35°C to improve detection of MRSA, and in the present study, all 51 MRSA isolates were readily detected at 36°C.

This study was not intended to issue general rAST guidelines. We rather used our local epidemiology as a paradigmatic example to investigate the challenges of rAST. Aggregated datasets from different geographies will be needed to develop general guidelines for rAST similar to current EUCAST procedures for CBP setting.[24] However, rapid disc diffusion data may be analysed in a systematic way:

(i) If non-WT strains cannot safely be discriminated from the WT population at early reading times, a grey zone covering the

diameter range of overlapping WT and non-WT populations may be introduced. Such grey zones would work as a buffer to prevent categorization errors but would also allow rapid reporting of a substantial number of isolates in the non-overlapping ranges as either susceptible or resistant. Of note, most problems with overlapping WT and non-WT populations found in this study were not limited to early reading times but were also present at standard reading at 18 h, e.g. amoxicillin/clavulanate or piperacillin/tazobactam and ESBL, or cefoxitin and PBP2a in *S. epidermidis*. The concept of technical buffer zones that has been demonstrated to prevent major and very major categorization errors for standard incubation, may thus be a reasonable option for rAST, too.[23]

(ii) If non-WT strains can safely be discriminated from the WT populations, it needs to be determined whether WT zone diameters and the corresponding ECOFFs shift during the incubation period (Figure 1).

(iii) If WT diameters and ECOFFs are stable, current EUCAST CBPs may readily be applied (34 of 44 species–drug combinations and Enterobacteriaceae in this study, i.e. 77.3%).

(iv) If ECOFFs shift during the incubation period, CBP adaptations may be necessary for early reading as shown for staphylococci that were displaying diameter increases >2 mm (the fifth percentile of the WT population served as a surrogate for the ECOFF; Figure 1).

In conclusion, this study demonstrated that: (i) the most important non-WT populations can be separated from the WT at early reading times; (ii) the same expert rules for standard incubation can be used for early reading; and (iii) for several species–drug combinations, particularly for staphylococci, CBP adaptations at early reading times may be necessary to ensure adequate categorization.

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Transparency declarations

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Supplementary data

Table S1 and Figure S1 are available as Supplementary data at *JAC* Online.

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